

EXTENDED REPORT

Nucleosomes possess a high affinity for glomerular laminin and collagen IV and bind nephritogenic antibodies in murine lupus-like nephritis

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Ann Rheum Dis 2007;**66**:1661–1668. doi: 10.1136/ard.2007.070482

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Accepted 30 April 2007
Published Online First
15 May 2007

Aim: Lupus nephritis is closely associated with in vivo autoantibody-binding to glomerular membrane-associated electron-dense structures (EDS). The biochemical nature and cellular origin of EDS are controversial, and definitive characterisation needs to be performed.

Methods: By using the terminal transferase biotin-dUTP nick end-labelling (TUNEL) assay at the electron microscopic level, we have traced extracellular chromatin within the glomerular basement membranes of nephritic (NZB×NZW)F1 mice. The TUNEL assay was subsequently used in combination with standard immune electron microscopy (IEM). To analyse why chromatin particles associate with membranes, we determined the affinity of nucleosomes and DNA for glomerular laminin, collagen IV and the mesangial matrix proteoglycan perlecan by surface plasmon resonance.

Results: This intra-assay colocalisation TUNEL IEM demonstrated that autoantibodies fully colocalised with extracellular TUNEL-positive chromatin observed as EDS in glomerular membranes, similar to results obtained by the same technique applied to human lupus nephritis. Most importantly, these data validate the murine variant of lupus nephritis as a model to study origin of extracellular chromatin as a key element in human lupus nephritis. Kinetic analyses demonstrated that nucleosomes had a high affinity for collagen IV and laminin, but not for perlecan.

Conclusion: Collectively, these results provide firm evidence that dominant target structures for nephritogenic autoantibodies are constituted by TUNEL-positive chromatin associated with glomerular capillary and mesangial matrix membranes at high affinity.

Systemic lupus erythematosus (SLE) is characterised by production of antibodies to DNA and nucleosomes. These are central in both a diagnostic and a pathogenic context.^{1–4} Of the organ manifestations in SLE, renal involvement is one of the most serious complications.⁵

Firm descriptions of glomerular target structures for nephritogenic autoantibodies are, although studied over decades, inconsistent. Two hypotheses dominate this field. Either nephritogenic autoantibodies recognise externalised nucleosomes associated with glomerular membranes,^{6–8} or they cross-react with non-nucleosomal glomerular antigens like laminin, collagen or α -actinin.^{9–12}

Recently, we have analysed murine nephritic kidneys by morphological and immunological assays, including immune electron microscopy (IEM) and colocalisation IEM.^{13–14} With these techniques, we have observed antibody-binding in vivo confined to electron dense structures (EDS) in glomerular membranes.¹⁴ These in vivo-bound autoantibodies fully colocalised with experimental monoclonal antibodies against dsDNA, histones or against transcription factors.¹⁴

Since the experimental monoclonal antibodies (mAbs) used in colocalisation IEM may be cross-reactive,^{9–12–15} it was decided to implement an independent DNA-specific assay, like the terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling (TUNEL) assay, to describe the nature of glomerular membrane-associated EDS. Furthermore, it became important to establish why such structures associate with glomerular membranes, to develop treatment modalities to avoid this potentially pathogenic association.

Data from the present analyses demonstrate that (1) autoantibodies are exclusively present in structures described 20–30 years ago as glomerular, membrane-associated EDS,^{16–18}

(2) the EDS contain TUNEL-positive DNA that colocalise perfectly with in vivo-bound antibodies and (3) nucleosomes bind renal capillary and mesangial matrix membrane collagen IV and laminin at high affinity. Perlecan, a glomerular mesangial matrix heparan sulfate proteoglycan (HSPG), did not bind nucleosomes in these experiments. From the present results, we revitalise the impact of nucleosomes and anti-nucleosome antibodies in lupus nephritis.

MATERIAL AND METHODS

Kidney preparation, antigens and antibodies

Kidneys from BALB/c and B/W mice (Harlan, Bicester, UK) were fixed in 8% depolymerised paraformaldehyde and epoxy-embedded (for TEM), or frozen in nitrogen (for IEM and TUNEL-IEM) as described previously.^{14–19} Sera were stored at –20°C until use. The project was approved by The Norwegian Ethical and Welfare Board for Research Animals, and treatment and care of the mice were in accordance with institutional guidelines.

Laminin was purchased from Biomedical Technologies (Stroughton, MA) and collagen IV from BD Biosciences (San Jose, CA). HSPG was obtained from Sigma-Aldrich (St. Louis, MO) and contained 460 µg of HS per 900 µg of core protein. Mass spectrometry analysis identified the HSPG to be perlecan (see below). This is in agreement with the HSPG produced in

Abbreviations: EDS, electron-dense structures; GBM, glomerular basement membrane; HSPG, heparan sulfate proteoglycan; IEM, immune electron microscopy; mAb, monoclonal antibodies; Nuc, nucleosomes; SLE, systemic lupus erythematosus; SN, stripped nucleosomes; SPR, surface plasmon resonance; TEM, transmission electron microscopy; TUNEL, terminal transferase biotin-dUTP nicked end-labelling

Engelbreth-Holm-Swarm mouse sarcoma cell.²⁰ Calf thymus dsDNA was obtained from Sigma-Aldrich, and mouse genomic dsDNA obtained from Calbiochem (Bad Soden, Germany). Antibodies to renal laminin were from Sigma-Aldrich, to collagen IV from MD Biosciences (St. Paul, MN), and to perlecan from Upstate Biotechnology (Lake Placid, NY). The anti-DNA 163p77 mAb was provided by Dr Tony Marion (University of Tennessee Health Science Center, Memphis, TN).

Nucleosome preparation

Stripped nucleosomes (SN), devoid of H1 and non-histone proteins, were kindly provided by Dr Burlingame (INOVA Diagnostics, San Diego, CA). Nucleosomes were prepared from a murine fibroblast cell line and characterised as described previously.^{21–22} SN was shown to contain the core histones H2A, H2B, H3 and H4, while Nuc contained all histone classes and several non-histone proteins. Both had DNA with a size distribution from 200 bp to several thousand bp (see fig 3C for details).

ELISA

Serum antibodies were detected by ELISA exactly as described in detail,^{4 14 23 24} using microtitre plates (Nunc MaxiSorp; Nunc, Copenhagen, Denmark) coated with nucleosomes (10 µg/ml as DNA) or calf thymus DNA (10 µg/ml).

Immune electron microscopy (IEM) and colocalisation IEM

Ultra-thin cryosections of glomeruli from BALB/c or nephritic B/W mice were prepared as described.^{13 19} In vivo-bound autoantibodies were detected by incubating the sections with rabbit antimouse (RaM) IgG antibodies followed by protein A-5nm gold conjugate (PAG-5nm). This procedure represents the IEM. For colocalisation IEM, the sections were then blocked with glutaraldehyde and glycine, and further incubated with the anti-DNA 163p77 monoclonal antibody (mAb), or an antilaminin β2 antibody, followed by incubation with RaM IgG and subsequently with PAG-10nm.¹⁴ Micrographs were taken at ×40 000 magnification using a JEM-1010 transmission electron microscope (Jeol, Tokyo)

Colocalisation TUNEL IEM assay

Extracellular chromatin was detected by an in situ end-labelling assay according to standard protocols with minor modifications.²⁵ In short, ultra-thin renal cryosections were incubated in terminal deoxynucleotidyl transferase (TdT) reaction buffer (RB, 25 mM Tris-HCl, 200 mM potassium cacodylate, 25 µg/ml BSA, 1 mM CoCl₂, pH 6.6) for 5 min, followed by a 60-min incubation at 37°C with 400 U of recombinant TdT (Roche Applied Science) in TdT-RB supplemented with 40 µM biotin-16-dUTP, in a humid chamber (Hybrite, Vysis, Abbot Molecular, Des Plaines, IL). Positive control sections were incubated with 3 U/ml DNase I in 40 mM Tris, 6 mM MgCl₂, pH 7.5, for 10 min at R.T. The reaction was stopped with 10 mM Tris-HCl, 1 mM EDTA prior to the TUNEL assay. Negative control sections were incubated in RB supplemented with biotinylated dUTP in the absence of TdT. Subsequently, the sections were incubated for 15 min in 1% fish skin gelatin (FSG). At this stage, the sections were incubated with RaM IgG antibodies for 20 min at room temperature to trace in vivo-bound autoantibodies, followed by incubation with PAG-5nm. The sections were then blocked with glutaraldehyde and glycine. Incorporated biotinylated nucleotides were labelled with a rabbit anti-biotin antibody for 20 min followed by PAG-10nm. The sections were washed in PBS between all incubations and finally contrasted with uranyl

acetate in methylcellulose, and examined using the JEM-1010 transmission electron microscope.

Surface plasmon resonance (SPR) analyses

SPR analyses were performed using the Biacore T100 system. Collagen IV, laminin and perlecan proteins were immobilised according to the manufacturer's instructions. Dextran (CM5) and Streptavidin (SA series S) coated sensor chips, Amine coupling kit, running buffer 10×HBS-EP+, immobilisation buffers and regeneration solutions were obtained from Biacore (Uppsala, Sweden).

In pilot experiments, laminin and collagen IV were effectively immobilised on CM5 chips, while perlecan did not bind to this chip, but bound SA chips after biotinylation (data not shown). Collagen IV and laminin (20 µg/ml each) were immobilised on the surface of individual CM5 chips at a flow rate of 5 µl/min, or coimmobilised (50 µg/ml total protein) on one CM5 chip. Immobilisation of collagen IV, laminin and collagen IV/laminin was stopped at 6000RU, 5600RU and 11 400RU, respectively. After immobilisation, CM5 chips were inactivated with 1 M ethanolamine-HCl. The reference flow cell for each ligand was treated in the same manner but without the protein. Perlecan was biotinylated using ProtoArray Mini-Biotinylation Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The biotinylated perlecan, 40 µg/ml in running buffer, was immobilised on an SA chip at a flow rate of 10 µl/min. The maximum obtained binding of perlecan was reached at 500RU. Binding of relevant antibodies to the 3 chips ascertained that the immobilised ligands were accessible at the surface, and that the antigenic structures recognised by relevant antibodies were preserved. These structures were therefore also regarded valid for studies of nucleosome interactions with the immobilised ligands.

Nucleosomes (Nuc and SN), DNA and antilaminin, anticollagen IV or antiperlecan antibodies were diluted twofold in running buffer from 500 nM (for nucleosomes, the concentration is given as mononucleosomes, defined as 200 kDa as the basic unit, while the concentration of DNA was calculated using 100 kDa for 200 bp) and injected over the surfaces with a flow rate of 5 µl/min for 900 s followed by injection of running buffer for 600 s. All responses were normalised and expressed relative to the baseline defined by the running buffer. Regeneration was performed with glycine-HCl, pH 1.5 (CM5 chip) or pH 3.0 (SA chip), at 30 µl/min for 2 min. Values from the reference flow cell were subtracted from all sensorgrams.

Analysis of the sensorgrams was performed with the Biacore T100 control software and Biacore T100 evaluation software 1.1. The association (k_a) and dissociation (k_d) constants were determined, and K_D was calculated, using the model for 1:1 ligand interaction.

RESULTS

Experimental validation of colocalisation TUNEL IEM for analyses in murine lupus nephritis

Control experiments and assay validation of TEM, IEM, colocalisation IEM and colocalisation TUNEL IEM, respectively, were performed on glomeruli of all mice included in this study, and are demonstrated in fig 1 for glomeruli of the nephritic mouse B/W15. TEM revealed mesangial and capillary membrane-associated EDS (fig 1A), and IEM demonstrated deposits of autoantibodies, perfectly localised within EDS (fig 1B). Colocalisation IEM analyses revealed that the anti-dsDNA 163p77 mAb and in vivo-bound autoantibodies colocalised in structures constituting EDS (fig 1C), confirming previous results on these kidneys.¹⁴ Antilaminin antibodies, on the other hand, bound glomerular membrane structures surrounding

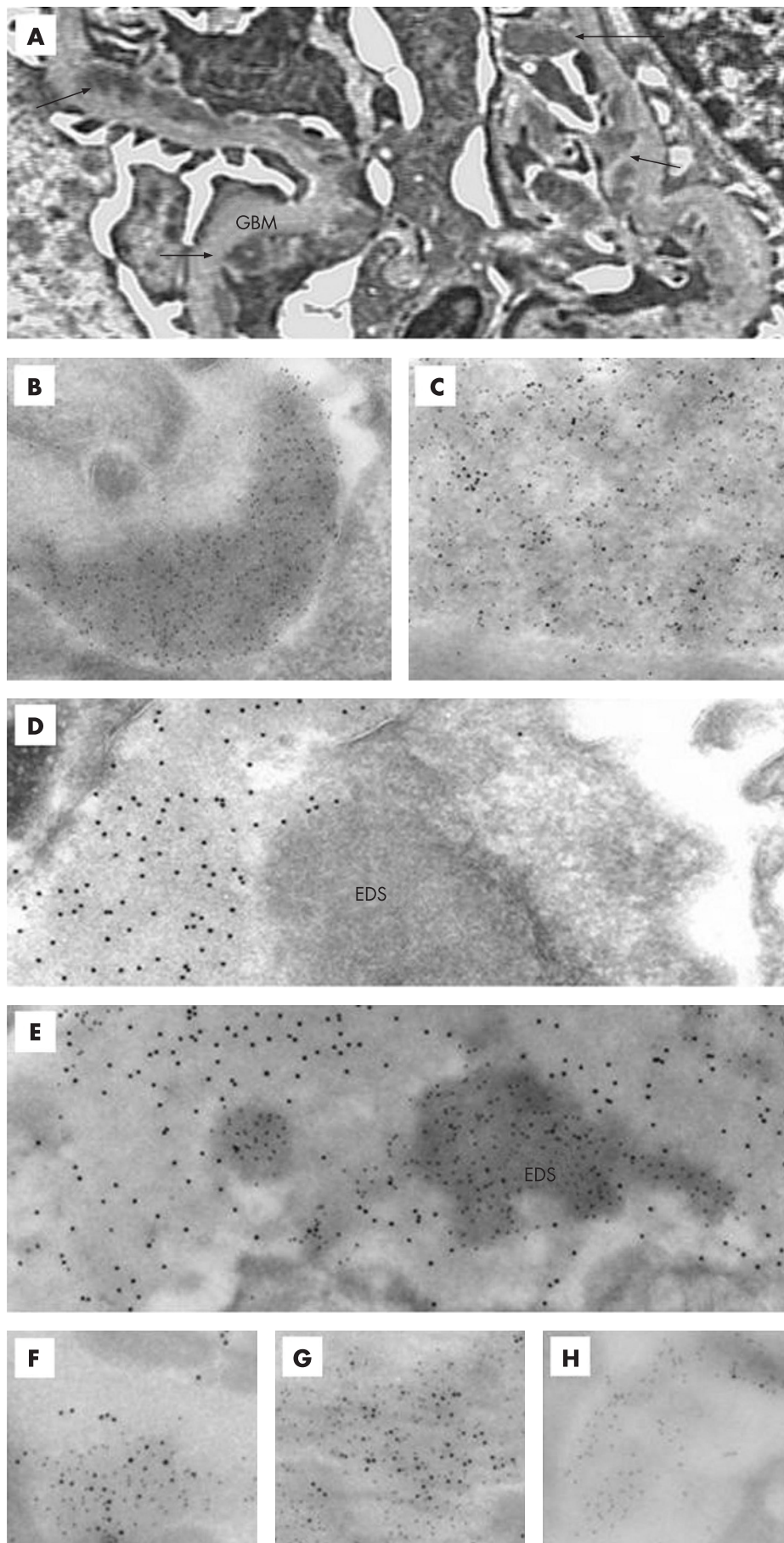


Figure 1 Validation of assays aimed at detection of glomerular deposits of autoantibodies and extracellular chromatin in nephritic B/W kidney sections by TEM, IEM, colocalisation IEM and colocalisation TUNEL IEM. TEM (A) and IEM (B) analyses revealed heavy mesangial and capillary membrane-associated EDS (arrows A), and in vivo-bound autoantibody deposits were strictly confined to EDS (B). Colocalisation IEM analyses demonstrate that anti-dsDNA 163p77 (PAG-10nm) and autoantibodies (PAG-5nm) colocalised in EDS (C). Rabbit antibodies to laminin (10 nm gold) stained regular membranes surrounding EDS (D), and this antibody did not colocalise with autoantibody deposits in EDS (5 nm gold) (E). In (F), colocalisation TUNEL IEM demonstrates that autoantibodies (PAG-5nm) and TUNEL-positive chromatin (PAG-10nm) colocalise in EDS. In (G), the results of DNase 1 digestion of glomerular section prior to colocalisation TUNEL IEM assay are demonstrated, while in (H), colocalisation TUNEL IEM assay with exclusion of TdT reveal no unspecific binding of PAG-10nm. GBM, glomerular basement membrane; EDS, electron dense structures.

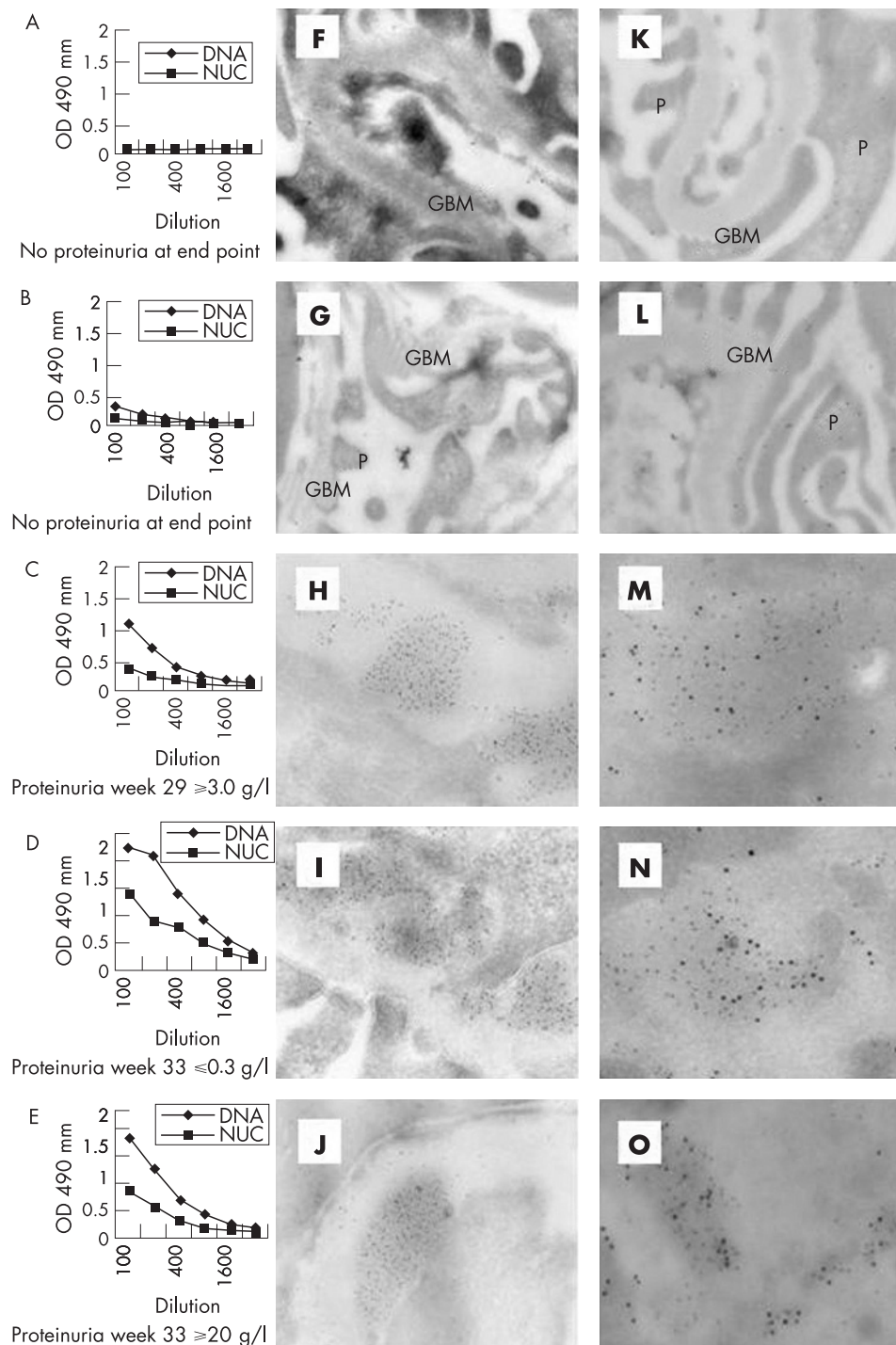


Figure 2 ELISA for detection of serum antibodies to dsDNA and nucleosomes, and IEM and colocalisation TUNEL IEM analyses of kidney sections. Results of these analyses are presented for kidney sections of BALB/c (A,F,K), B/W52 (B,G,L), B/W2 (C,H,M), B/W15 (D,I,N) and B/W16 (E,J,O). (A–E) Titration of serum anti-DNA and antinucleosome autoantibodies and information on proteinuria at the end point (see Kalaaji *et al*¹⁴ for details); (F–J) IEM to trace autoantibody deposits (PAG-5nm) of BALB/c, B/W52, B/W2, B/W15 and B/W16, respectively; (K–O) colocalisation TUNEL IEM to analyse whether autoantibodies bound in vivo colocalise with TUNEL-positive glomerular membrane-associated structures in BALB/c, B/W52, B/W2, B/W15 and B/W16, respectively. Proteinuria was determined by stix from Bayer Diagnostics (Bridgend, UK): 0–1+, ≤ 0.3 g/l, regarded as physiological proteinuria; 2+, 1 g/l; 3+, ≤ 3 g/l; and 4+, ≥ 20 g/l. GBM, glomerular basement membrane; P, podocytes.

EDS (10 nm gold, fig 1D and 1E), while autoantibodies bound EDS (5 nm gold), as shown by colocalisation IEM (fig 1E). By colocalisation TUNEL IEM, autoantibodies were detected in EDS that contained material stained by the TUNEL assay (fig 1F). DNase 1 treatment prior to TUNEL assay resulted in increased labelling of chromatin in EDS (fig 1G), but did not uncover further extracellular chromatin (not shown). Colocalisation TUNEL IEM assay in absence of TdT resulted in detection of autoantibodies, while antibiotin antibodies and PAG-10nm gold conjugate did not bind non-specifically (fig 1H). These data indicate that murine and human lupus nephritis have a common genesis placing nucleosomes as a key element in both.²⁶

Analyses of serum antibodies to dsDNA and nucleosomes, and determination of glomerular antibody deposits and their colocalisation with TUNEL-positive extracellular chromatin

Selected for the present studies are sera and kidneys from a control BALB/c mouse, from the 10-week-old non-nephritic B/W52 mouse, or from 3 B/W mice producing antibodies to dsDNA and nucleosomes (B/W2, B/W15 or B/W16). Figure 2A–E demonstrate antibody titration at week 33 (end point) of BALB/c, B/W52, B/W2, B/W15, B/W16, respectively. The B/W2, B/W15 and B/W16 mice, but not BALB/c or B/W52, produced anti-dsDNA and antinucleosome antibodies. Of these, B/W2 and B/W16, but not B/W15, had proteinuria.

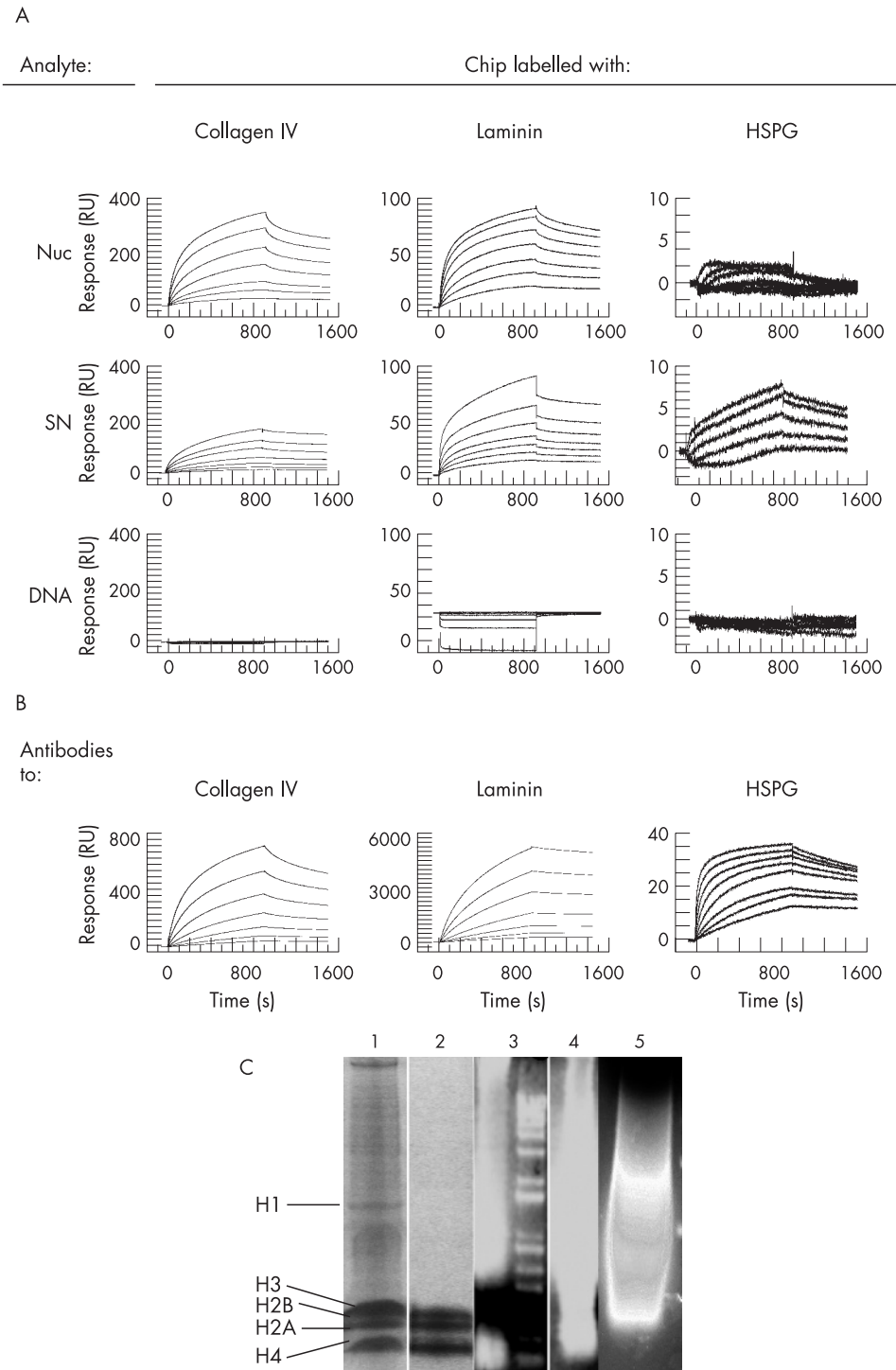


Figure 3 Surface plasmon resonance (SPR) analyses of binding of nucleosomes to glomerular membrane structures. Sensorgrams are presented for binding analyses of nucleosomes (Nuc), stripped nucleosomes (SN) and DNA (A) or antibodies (B) at different concentration (twofold dilution from 500 nM) to immobilised collagen IV, laminin or perlecan. Nucleosomes (Nuc), stripped nucleosomes (SN) and mouse genomic DNA, used for studies of interaction with the immobilised ligands laminin, collagen IV or perlecan, are characterised by SDS-PAGE and ethidium bromide-stained 1% agarose gels (C). Nuc is demonstrated by SDS-PAGE to contain all histone classes and several non-histone proteins (lane 1), while SN contained the core histones only (lane 2). Both preparations had a DNA size distribution from approximately 200 to several thousand bp (lanes 3 and 5 for Nuc and SN, respectively; lane 4 present 1 kb+ DNA ladder). The size of the mouse genomic DNA used as analyte for binding studies is shown in lane 6.

IEM analyses of glomeruli of BALB/c and B/W52 revealed no autoantibody-binding in the glomeruli (fig 2F,G, respectively), consistent with their normal appearance without EDS. Glomeruli of the other mice (B/W2, B/W15 and B/W16) were characterised by accumulation of EDS in glomerular membranes, and antibody deposits were strictly confined to these EDS (fig 2H,I,J, for B/W2, B/W15 and B/W16, respectively). Further details on glomerular pathology and apoptosis in these kidneys are presented in Kalaaji *et al.*¹⁴

Glomerular membranes of BALB/c and B/W52 did not contain TUNEL-positive material (fig 2K,L for BALB/c and B/W52, respectively). Colocalisation TUNEL IEM applied to glomeruli of B/W2, B/W15 and B/W16 demonstrated the

presence of TUNEL-positive (10 nm gold) membrane-associated EDS that were targeted in vivo by autoantibodies (5 nm gold) (figs 1F and 2N for B/W15, fig 2M,O for B/W2 and B/W16, respectively). DNase I digestion of the same samples showed increased labelling of chromatin in EDS within glomerular membranes but did not uncover further extracellular DNA (data not shown, exemplified in fig 1G for B/W15). Colocalisation TUNEL IEM performed in the absence of TdT resulted in detection of autoantibodies (5 nm gold), but not of biotinylated nucleotides, in EDS (fig 1H for B/W15). These results reveal that membrane-associated EDS in nephritic glomeruli contain TUNEL-positive DNA targeted by nephritogenic autoantibodies.

Binding of nucleosomes to immobilised glomerular laminin, collagen IV or perlecan

To understand why nucleosomes redistribute to glomerular capillary and mesangial matrix membranes, we tested binding of nucleosomes or purified DNA to three major membrane components, collagen IV, laminin and perlecan by SPR analyses.

Control liquid chromatography/mass spectrometry (LC/MS, nanoAquity (Waters, Milford, MA) and Q-TOF Ultima global mass spectrometer (Micromass, Manchester, UK)) analyses of the ligands immobilised on Biacore chips demonstrated that the laminin preparation contained laminin $\beta 2$ and also $\alpha 1$ chains, while HSPG was demonstrated to contain predominantly perlecan, and traces of laminin $\alpha 1/\beta 2/\gamma 1$. The recombinant collagen IV was pure according to SDS-PAGE (data not shown).

Serial dilutions of Nuc, SN, DNA, and of antibodies to laminin, collagen IV or perlecan were injected over the chips with immobilised membrane components. The response values at steady states and the dose-response of the analytes are presented in fig 3A,B. The sensorgrams of laminin or collagen IV chips demonstrated binding of Nuc and SN, but not of DNA (fig 3A). The sensorgrams of perlecan showed no binding of Nuc or DNA, and only marginal binding of the artificial SN (fig 3A). Antibodies to collagen IV, laminin and perlecan bound the ligands at relatively high affinities ($K_D < 5.1 \times 10^{-8}$, table 1, fig 3B). The differences in RU units between the antibodies reflect most probably the differences in the number of molecules immobilised on the chips and the numbers of determinants recognised by individual antibodies.

The k_a , k_d and K_D values of Nuc- and SN-binding to the collagen IV or laminin chips were calculated, and the results are listed in table 1. In general, Nuc possessed a higher affinity for laminin ($K_D = 9.7 \times 10^{-9}$) than for collagen IV ($K_D = 3.2 \times 10^{-8}$), while Nuc possessed almost a 100-fold higher affinity than SN for laminin (K_D 0.966×10^{-8} M and K_D 1.0×10^{-8} M, for Nuc and SN respectively). This difference may be caused by interaction with laminin by non-histone proteins presented by Nuc that are absent in SN (see fig 3C for details). As Nuc did not bind perlecan, and the artificial SN demonstrated marginal binding to perlecan, affinities were not calculated for this ligand.

To test if laminin and collagen IV together influenced the binding of Nuc, equimolar amounts of laminin and collagen IV were immobilised on the same chip (table 1). The binding of Nuc to this chip was lower ($K_D = 1.8 \times 10^{-8}$) compared with binding to chips presenting individual molecules. The affinities calculated for binding of individual antilaminin and anticollagen IV antibodies to the dually coated chip were also slightly lower than for the individually coated chips (table 1).

DISCUSSION

In this study, analyses were performed to determine the nature of glomerular, membrane-associated EDS, and to determine why EDS associate with capillary and mesangial matrix membranes in lupus nephritis. The present results demonstrate that EDS contain nucleosomes and that these represent target structures for nephritogenic antibodies *in vivo*,^{6 14 27} and contradict the fact that nephritic, *in vivo*-bound autoantibodies recognise glomerular, non-nucleosomal antigens like laminin, collagen or α -actinin.^{9-12 15} Furthermore, we provide evidence that nucleosomes are redistributed to glomerular membranes due to a high affinity at least for glomerular laminin and collagen IV. This explains the distinct membrane-associated localisation of extracellular chromatin particles, and corresponding autoantibody deposits confined to these structures.

Dual specificity of anti-DNA antibodies for several inherent glomerular constituents have been reported.^{11 28-34} There is, however, no firm information as to the nature of the glomerular target structures that *de facto* have bound these antibodies *in vivo*, whether nucleosomes,³⁵ laminin,²⁸ or, for example, α -actinin^{31 32} or other antigens.

We have recently provided new information that may help solve this problem. With IEM, Kalaaji *et al* demonstrated that glomerular autoantibody deposits are associated with EDS characteristic of nephritic glomerular membranes.¹⁴ Furthermore, experimental antibodies to dsDNA, histones and transcription factors colocalised with *in vivo*-bound autoantibodies in EDS in capillary and mesangial matrix membranes.¹⁴ The localisation of membrane components like collagen IV and laminin were distributed around the EDS, and α -actinin was not a distinct part of EDS.¹³ This is consistent with observations that HSPG, laminin and collagen IV are not part of EDS in immune complex glomerulonephritis, as described by Moss *et al*¹⁶ and Haramoto *et al*.³⁷ However, if membrane constituents bind nucleosomes, they formally participate in the structures that phenomenologically are observed as EDS with EM, while still nucleosomes represent targets for nephritic antibodies.

Because of the need for new therapy modalities for lupus nephritis, the decision was made to obtain data that conclusively define the nature of EDS that are targeted by nephritic antibodies *in vivo*. We therefore developed the colocalisation TUNEL IEM assay. Results clearly demonstrated that membrane-associated EDS contain TUNEL-positive DNA in the form of nucleosomes, and that these structures are targeted by nephritic autoantibodies *in vivo*. These data validate and confirm the less reliable data obtained in colocalisation IEM using potentially cross-reactive experimental monoclonal antichromatin antibodies (data summarised in table 2). Since the present results are similar to those obtained in human lupus nephritis,²⁶ they validate the murine lupus nephritis model for further studies on the basic nature of human lupus nephritis.

Table 1 Determination of affinity of nucleosomes and antibodies for glomerular collagen IV and laminin by surface plasmon resonance analysis

Ligand on chip	Analyte	k_a (10^3 /M/s)	k_d (10^{-4} /s)	K_D (10^{-8} M)	χ^2 (RU ²)
Collagen IV	Nuc*	12.1	3.896	3.201	104
	SN†	7.553	1.293	1.712	30.0
	Anticollagen IV	8.116	4.157	5.122	241
Laminin	Nuc	44.56	4.306	0.967	29.0
	SN	12.51	1.285	1.027	23.2
	Antilaminin	6.086	0.521	0.855	4.95×10^3
Collagen IV/laminin	Nuc	9.789	1.775	1.813	8.83
	Antilaminin	7.991	0.448	0.561	1.52×10^4
	Anticollagen IV	12.89	2.151	1.669	71.1

*Nuc, eukaryotic, non-stripped nucleosomes generated by micrococcal nuclease digestion. †SN, stripped nucleosomes.

Table 2 Summary of results of experiments addressed to determine the nature of electron dense structures associated with glomerular membranes in nephritic kidneys that are targeted by autoantibodies in vivo

Constituents	Healthy glomeruli	Structures characterised in glomerular membrane-associated electron dense structures			References	Interpretation of EDS composition
		Nephritic glomeruli (NZB×NZW)F1	Present in membranes outside EDS	Human lupus nephritis		
DNA	Not detected	Dense pattern confined to EDS	Negative	Dense pattern in EDS	14 26 42	Presence of DNA, histones and transcription factors indicates accumulation of nucleosomes in EDS associated with membranes
Histones	Not detected	Dense pattern confined to EDS	Negative	Dense pattern in EDS	14 26 42	
Transcription factors*	Not detected	Presence confined to EDS	Negative	Present in EDS	14 26	
Laminin	Normal membrane constituent	Surrounding EDS†	Normal pattern	Not analysed	14	Laminin and collagen are not part of EDS
Collagen	Normal membrane constituent	Surrounding EDS	Not analysed	Not analysed	14	
α-Actinin	In podocytes and mesangial cells	Present in membranes, interstitium and in EDS	Random pattern in the extracellular space	Not analysed	13	α-Actinin is extracellular in nephritic glomeruli

*To demonstrate the presence of transcription factor in EDS, antibodies to Tata-box binding protein (TBP) have been used, as this factor is constitutively bound to nucleosomes; †as an example of this, see fig 1D,E.

Whether nucleosomes are trapped in glomerular membranes, like fish in a net, or inherit particular affinities for glomerular components has not been determined previously. DNA itself has been shown to have an affinity for collagen and collagen-like structures in glomerular membranes,³⁸ although we could not confirm this in our SPR analyses. Since DNA in vivo is present in the nucleosomal form, the binding properties of nucleosomes for glomerular membranes, for example, may be totally different from those of DNA. Berden and his group have investigated the basis for in vivo binding of anti-DNA antibodies to glomerular membranes. They observed that anti-DNA antibodies in complex with nucleosomes bound glomerular membranes and proposed that the binding was due to interaction of positively charged C- and N-terminal histone tails with negatively charged residues on agrin or collagen IV, for example.^{6 35 39 40} This has also been discussed by Lefkowitz and colleagues.^{7 27 41}

In this study, we investigated whether nucleosomes have an affinity for the main components of glomerular membranes by SPR. For these analyses, we used nucleosomes stripped for non-histone proteins, and nucleosomes generated by micrococcal nuclease digestion of chromatin,²² with structures and a size distribution similar to apoptotic nucleosomes as they appear in the context of apoptosis.

From SPR analyses, we observed binding of nucleosomes to the glomerular membrane components laminin and collagen IV, but not to perlecan. The results demonstrated furthermore that nucleosomes bind laminin and collagen IV with affinities equivalent to those of the control antibodies to laminin or collagen IV (see table 1 for details). For the calculations of k_a and k_d for nucleosome binding, we used the model for 1:1 ligand interaction. The observed curves gave a good fit with this model and allowed us to determine relative affinity differences between the analytes.

That nucleosomes did not bind perlecan was somewhat surprising, since perlecan have up to 4 HS chains,⁴³ and cationic N-terminal histone tails are assumed to bind to anionic HS in the glomerular basement membranes. Indeed, Kramers *et al*³⁵ and Berden *et al*⁴⁰ have demonstrated that the removal of HS chains from agrin can prevent binding of nucleosomes. The HSPG preparation used in these SPR analyses was prepared from Engelbreth-Holm-Swarm sarcoma cells and was demonstrated to contain predominantly perlecan heparan sulfate. Perlecan heparan sulfate is abundant in the glomerular mesangial matrix.⁴⁴ Glomerular extracellular nucleosomes are also located in the mesangial matrix, but the data from the SPR analyses indicate that perlecan is not the membrane structure

that binds chromatin. It will be interesting to determine whether agrin differs from perlecan in the ability to bind nucleosomes, since agrin is the most abundant HSPG in capillary membranes, and chromatin particles are also associated in these membranes. Nevertheless, it is quite clear from the present results that chromatin particles bind glomerular membranes at least through their high affinity for glomerular laminin and collagen.

The generation of nucleosomes in vivo requires apoptosis,⁴⁰ and the disordered regulation of both apoptosis and the clearance of apoptotic products has been proposed to be implicated in the pathogenesis of SLE and lupus nephritis.⁴⁵⁻⁴⁷ An early event in lupus nephritis may therefore be the release of apoptotic nucleosomes that may bind components of glomerular membranes at high affinity. It is imperative in the context of present and previous results to analyse the factors that affect apoptosis in SLE.

ACKNOWLEDGEMENTS

This project has been financed by grants from the Norwegian Foundation for Health and Rehabilitation through the Norwegian Rheumatology Organization, by the Milieu Support given to OPR from the University of Tromsø and by grants from Northern Norway Regional Health Authority Medical Research Program. We thank Randi Olsen, Department of Electron Microscopy, IMB, University of Tromsø, for excellent technical assistance. Dr Tony Marion generously gave us the 163p77 anti-DNA mAb. Dr Rufus Burlingame kindly provided us with stripped nucleosomes. We thank Manar Kalaaaji for providing us with ELISA data on sera and the kidneys necessary for this study.

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Competing interests: None declared.

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